AUTOLYSIS OF THE GONOCOCCUS.*

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IN 1899 Emmerich and Low[1] reported results of extensive experiments in which they called attention to the presence and action of a ferment in *B. pyocyaneus*. According to their reports, they were able to recover this ferment in appreciable amount and with this material in a concentrated form secured results which indicated that the ferment was destructive to a number of species of micro-organisms, among which was included the gonococcus.

Flexner[2] demonstrated that the vitality of the meningococcus was very materially lessened and the germs themselves partially dissolved when they were allowed to remain in suspension in salt solution. He found the products of the disintegration to be detrimental to a luxuriant development of that organism on the medium upon which they had grown and on a fresh medium to which some of the products of disintegration had been transferred.

A biological comparison of the gonococcus and *M. intracellularis* by Martha Wollstein[3] substantiates Flexner's earlier work and indicates that a ferment of decided activity is present in or about the micrococcus of gonorrhea. In producing an antigen Torrey[4] suspended a large and luxuriant growth of the gonococcus (in salt solution) and after submitting this suspension to a constant agitation for a number of hours, he filtered. The resulting clear filtrate was decidedly toxic.

The work of Wollstein, Torrey, and others indicates that a cell destruction occurs in cultures of the gonococcus when placed under certain conditions, the most important of which are temperature, concentration of medium, and age of culture.

With the supposition well established that autolysis of the gonococcus occurs under certain conditions it was the object of our work: (1) To test the results obtained by others; (2) to measure the extent of autolysis and amount of autolytic ferment present; (3) to determine the effect of autolysate on the growth of the gonococcus

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[1] *Ztschr. f. Hyg.*, 1899, 31, p. 1.

[2] *Jour. Exper. Med.*, 1907, 9, p. 105.

[3] *Ibid.*, 1907, 9. p. 588.

[4] *Jour. Med. Res.*, 1907, 16, p. 329.

and other organisms in vitro; and (4) from results of the first three to note the effect of a concentrated autolysate on the organism in vivo and its possible therapeutic application.

When the isolation and cultivation of the gonococcus was first undertaken by one of us (Clark), it was observed that considerable variation occurred in the depth of stain taken by the cocci and later that a suspension of the gonococcus in salt solution became appreciably less dense when left at room temperature for 12 to 24 hours. The loss in density was at first attributed to sedimentation. The changed character of the sediment especially noticeable in its tenacity and the observation of numerous fragmentary cell outlines under the microscope led us to conclude that the cocci had disintegrated at a rate far greater than was to be expected in an isotonic solution.

To obtain a point in departure from which loss in staining qualities, supposedly due directly or indirectly to changes in cell content, and the possible disintegration of the cells could be measured, an attempt was made to prepare a suspension of the gonococcus which would show none of these changes, the lack of such changes being determined by the unaltered appearance of the stained germs under the microscope and the maintenance of a constant bacterial count.

Staining qualities (*qualitative tests*).—A heavy suspension was secured from 20-hour cultures of the gonococcus on ascitic agar. This suspension was divided into two equal portions; to one was added 4 per cent trikresol and then both were sealed up in 1 c.c. amber glass bulbs. These lots were again subdivided into portions; one portion each being set aside without heating it and the remaining portions being subjected to varying degrees of heat for one hour and kept in the dark at room temperature. At the end of one and five days respectively a sample bulb from each lot was well shaken, opened, and a loopful of the suspension stained with methylene blue.

TABLE 1.

Stain Made	Without Trikresol				4 per cent Trikresol			
	No Heat	50° C. 1 hour	60° C. 1 hour	70° C. 1 hour	No Heat	50° C. 1 hour	60° C. 1 hour	70° C. 1 hour
After 24 hrs.	none	very few	none	numerous	none	very few	numerous	numerous
" 5 days	"	none	very few	"	"	none	"	"

Suspension contained 3,388,000,000 germs per c.c.

The results show:

1. Gonococci in suspension receiving neither heat nor trikresol lose completely their original staining qualities.

2. Trikresol alone preserves the staining qualities to a limited extent only.

3. In the presence of trikresol, the preservation of the cells is proportional to the amount of heat to which they are subjected; 60° C. and above for one hour causes them to retain their staining qualities almost perfectly.

4. In the absence of trikresol a greater degree of heat is required to accomplish the same result, 60° C. for one hour with trikresol being equivalent to 70° C. for one hour in the absence of trikresol.

We thus found that a permanent suspension so far as staining qualities are concerned can be secured by submitting the original suspension to 60° C. for one hour in the presence of trikresol or 70° C. for one hour in the absence of trikresol.

Bacterial counts (*quantitative*).—A heavy suspension of the gonococcus was prepared and immediately counted. It was allowed to remain at room temperature and counted at intervals during the succeeding 24 hours. Technique employed was similar to that described by Miller[1] in standardizing bacterial vaccines. Fresh blood from a small puncture in the finger is drawn into a blood-counting pipette to the first small division; a bubble of air is then admitted and one division of the suspension to be counted is taken in; the two equal portions are then thoroughly mixed on a clean slide; and finally small drops are placed on clean slides and spread as for making a differential blood count. Slides are then stained two minutes with Hastings' stain and the bacteria and red blood cells in a given number of fields counted; the number of germs per c.c. is concluded from these results basing the known factor on the number of red blood cells found per c.c. of blood used in making the count.

From a number of tests made the following is given as typical of results obtained:

TABLE 2.

	Time of Counts					
	9:00 A. M.	9:30 A. M.	10:00 A. M.	2:00 P. M.	5:00 P. M.	8:30 A. M. (23½ hrs. later)
Result of Counts..	336,575,000	heated 50° C. ½ hr.	244,540,000	139,000,000	135,540,000	113,000,000
Result of Counts..	180,000,000	heated 70° C. ½ hr.	83,000,000	104,000,000	83,000,000	89,000,000

These results are summarized as follows:

1. In one-half hour at room temperature before heating approximately 28 per cent of the cocci lost the power to take the stain.

2. During the following 22½ hours after heating at 50° C. for 30 minutes, a further 38 per cent of its original number lost their staining

[1] *Ther. Gaz.*, 1907, 23, p. 173.

power, making a total loss of 65 per cent; the decrease, however, being most rapid in the first five hours.

3. A gradual decrease in the number of staining organisms is observed after submitting the suspension to 50° C. for 30 minutes.

It is clearly shown from the table and summaries that a temperature of 50° C. for one-half hour does not prevent disintegration. Subsequent to this test, a suspension was counted, subjected to a temperature of 70° C. for one-half hour, and recounted at intervals. The suspension employed again showed a decided loss during the interval between taking up and heating. After heating at 70° C. for one-half hour no decrease in the number of stained organisms was observed, counts revealing the same number after one and 18 hours respectively.

These results confirm the simple staining results by showing that 70° C. for one-half hour prevents all disintegration and produces a permanent suspension and adds to these results the rate at which disintegration occurs.

Having thus demonstrated to our entire satisfaction that a change occurs in the cell which robs it of its original characteristics, questions presented themselves: (*a*) Is the loss of staining qualities due to a diffusion of contents through the cell wall? (*b*) Is the change within the cell wall itself? or (*c*) Does a break in the cell wall allow the contents to escape into the surrounding liquid?

We found that a thoroughly washed, 24-hour culture of the gonococcus after standing in salt solution for a time liberated sufficient quantities of proteid to give a precipitate with dilute acetic acid and heat. Making use of this fact as a basis for future work considerable progress was made toward a solution of the problems in question.

Large amounts of the gonococcus were grown on ascitic agar and taken up in a small quantity of salt solution. A perfect suspension was produced by shaking, the germs being thrown down by centrifugalizing, the supernatant liquid removed, and the germ residue again suspended in salt solution. This process was repeated until the Berkefeld filtrate from the supernatant liquid was free from proteid as shown by failure to precipitate with acetic acid and heat. After making sure by this method that all traces of the culture medium and soluble proteid were removed, the suspension of germs was divided into portions and allowed to stand at different temperatures. After 6, 18, and 24 hours, the suspensions were again centrifugalized and the supernatant liquid filtered and tested for proteid.

The technique described was repeated on several lots of suspension with decidedly uniform results. The portion kept at room temperature for lengths of time varying from 6 to 24 hours gave a heavy precipitate with dilute acetic acid and heat. The portion heated at 70° C. for one-half hour did not give a precipitation with acetic acid and heat, which fact alone substantiates earlier findings.

These results are in themselves conclusive. Products of change can be detected in the suspending fluid by chemical means. Just what is the nature of this change is not, however, proven by this experiment. It may be partial disintegration of cell wall or diffusion of cell contents through cell wall. From frequent microscopical studies of the partially autolysed suspensions, we are led to favor the first assumption. Numerous irregular, imperfect outlines of the coccus are found to have taken the stain normally, while the ordinarily readily stained interior remains unchanged; consequently, we conclude that a partial disintegration of the cell wall sufficient to liberate contents occurs, although not extensive enough to destroy the staining properties of the wall.

ACTION OF ALCOHOL ON AUTOLYSIS.

Those phases of the work dealing with the effect of heat and preservative on the staining qualities of the gonococcus reveal the fact that heat at 70° C. preserves staining qualities, that is, prevents disintegration. The effect of alcohol in varying dilutions on suspensions of the gonococcus was tested as follows:

Four drops of a fresh suspension were placed in tubes of normal solution containing 2 c.c. of 95 per cent, 50 per cent, 25 per cent, 10 per cent, 5 per cent, and no alcohol respectively. Every two hours one loopful of suspension from each tube was stained two minutes with Löffler's methylene blue and a comparison made of slides thus obtained during 24 hours. At the time of taking suspension for smears, a comparative record of its density was also noted. In tubes containing 95 per cent and 50 per cent alcohol, the bacteria rapidly settled to the bottom owing to the low specific gravity of the fluid, but slight agitation restored the suspension, showing that true proteid precipitation had not occurred.

The results are shown in Table 3.

Briefly, alcohol in 50 per cent and 95 per cent dilutions preserves the staining qualities of the germs. In lower dilutions changes occurred, as is shown by loss in density and by imperfect staining in all tubes except those containing the two highest percentages of alcohol.

At this stage of our investigations, after we had proven that a rapid change occurs in suspension of the gonococcus (autolysis), when left under ordinary room conditions, and that such changes can be controlled and prevented at will by the use of heat, trikresol, and alcohol, it became desirable to determine the effect of such disintegration products upon fresh, active cultures of the gonococcus on fresh culture media.

TABLE 3.

Suspending Fluid		Time of Examinations				
		9:30 A. M.	11:15 A. M.	1:15 P. M.	3:15 P. M.	10:00 A. M. ($24\frac{1}{2}$ hrs. later)
Alcohol 95 per cent	Sediment	heavy	heavy	heavy	heavy	heavy
	Precipitations	none	slight	slight	slight	slight
	Stain	deeply	deeply	deeply	deeply	deeply
Alcohol 50 per cent in NaCl sol.	Sediment	heavy	heavy	heavy	heavy	heavy
	Precipitations	none	slight	slight	slight	slight
	Stain	deeply	deeply	deeply	deeply	deeply
Alcohol 25 per cent in NaCl sol.	Sediment	medium	heavy	slight	none	slight
	Precipitations	none	none	none	none	none
	Stain	deeply	medium	medium	o	faint
Alcohol 10 per cent in NaCl sol.	Sediment	slight	heavy	slight	slight	slight
	Precipitations	none	none	none	o	none
	Stain	medium	deeply	medium	o	very faint
Alcohol 5 per cent in NaCl sol.	Sediment	none	slight	none	slight	none
	Precipitations	none	none	none	none	none
	Stain	medium	medium	medium	very faint	very faint
NaCl sol.	Sediment	none	none	none	none	none
	Precipitations	none	none	none	none	none
	Stain	deeply	medium	faint	faint	none

An autolysate was prepared by macerating a large amount of growth of the gonococcus in a small amount of salt solution. The bottle containing this heavy suspension and a number of small glass beads was placed in a mechanical shaker and shaken continually for six hours. Tests on the autolysate proved it to be sterile after 24 hours. A few drops of this sterile material were flowed over the surface of inclined ascitic agar, Thalmann's agar, and plain agar. In 48 hours at incubator temperature the autolysate had become entirely absorbed by the agar, at which time the tubes, together with the control tubes, were planted with equal quantities of a gonococcus suspension taken directly from a luxuriant, viable, 24-hour culture. All tubes were incubated for 48 hours and the effect of the autolysate on the growth of the germs noted.

This experiment was repeated several times and from results obtained we are able to make the following statements.:

1. The autolysate on ascitic agar lessened the amount of growth by more than one-half over that obtained on untreated media.

2. When added to Thalmann's agar, the autolysate had the effect of completely inhibiting the growth.

3. In one set of tests, the gonococcus grew on plain agar alone, while the presence of the autolysate completely prevented development.

CONCLUSIONS.

Disintegration or autolysis occurs in gonococcus cultures to a marked degree.

This autolysis can be completely prevented by heat alone at 70° C. for one hour, by heat in the presence of trikresol at 60° C. for one hour, by 50 per cent and 95 per cent alcohol in salt solution, and partially by 0.4 per cent trikresol alone.

Specimens stained at certain stages of disintegration show numerous fragmentary cell walls still capable of taking the stain, indicating that the cell wall is not completely disintegrated.

The presence of shadow forms in which the cell wall only is stained indicates that the cell contents are either so changed as to be incapable of taking the stain or have escaped from within the wall.

The presence of soluble proteid substances (as shown by their precipitation with acetic acid) in the surrounding liquid indicates that the contents escape from within the cell.

Hence, it would seem that autolysis of the gonococcus is effected by rupture of the cell wall and escape of the contents.

The products of this autolytic process markedly inhibit the growth of the gonococcus on artificial culture media. Their use in combating the disease in man will form the subject of a future communication.